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Genetic diversity of *Hieronyma alchorneoides* (Phyllanthaceae) using Amplified Fragment Length Polymorphisms (AFLP) in Costa Rica

Heidy M. Villalobos-Barrantes[1,2*]; https://orcid.org/0000-0003-3657-4439
Federico J. Albertazzi[1,3]; https://orcid.org/0000-0002-8488-3941

1. Centro de Investigación en Biología Celular y Molecular (CIBCM), Universidad de Costa Rica, San José, Costa Rica; heidy.villalobosbarrantes@ucr.ac.cr (*Correspondencia).
2. Escuela de Química (EQ), Universidad de Costa Rica, San José, Costa Rica; heidy.villalobosbarrantes@ucr.ac.cr
3. Escuela de Biología (EB), Universidad de Costa Rica, San José, Costa Rica; federico.albertazzi@ucr.ac.cr

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ABSTRACT

Introduction: *Hieronyma alchorneoides* (Pilón) is a threatened and economically important timber species in the wet forest of Costa Rica.

Objectives: To assess the extent of the genetic diversity between and within populations of *H. alchorneoides* in Costa Rica.

Methods: A total of 245 samples were obtained from five regions from protected areas, secondary forest and pastures in Costa Rica. These were analyzed by amplified fragment length polymorphism (AFLP). AFLP were used to measure genetic diversity with % polymorphic loci, Shannon diversity index, population structure (AMOVA) and a principal coordinate analysis (PCA). An UPGMA dendrogram based on neighbor-joining cluster was performe.

Results: Five primers were used to generate 213 polymorphic bands. Molecular variance indicated a 73 % within locations with a PhiPT (φ st) of 0.266 and an expected heterozygosity from 0.298 to 0.363. The PCA showed five different groups: Northeast, Northwest, East, Central and Southwest. The AMOVA confirmed the assignment of the five groups, but the dendrogram arranged the sites in three groups: individuals from the Northeast and Northwest, individuals from the Central and Southwest, and individuals from the East.

Conclusions: The results suggest that *H. alchorneoides* has a wide range distribution in Costa Rica with a long-distance gene flow that keeps maintaining the genetic diversity between regions. Further research must be accomplished to enrich the information generated by this study.

Key words: AFLP, Phyllanthaceae, genetic diversity, tropical rain forest.

RESUMEN

Diversidad genética de *Hieronyma alchorneoides* (Phyllanthaceae) utilizando fragmentos amplificados polimórficos de longitud larga (AFLP) en Costa Rica

Introducción: El Pilón *Hieronyma alchorneoides* es una especie maderable amenazada y económicamente importante del bosque húmedo de Costa Rica.

Objetivos: Evaluar el grado de diversidad genética entre y dentro de las poblaciones de *H. alchorneoides* en Costa Rica.

Métodos: Se obtuvieron 245 muestras en cinco regiones de áreas protegidas, bosque secundario y pastizales de Costa Rica. Se analizaron mediante polimorfismo de longitud de fragmentos amplificados (AFLP). Se utilizaron AFLP para medir la diversidad genética con el % de loci polimórficos, índice de diversidad de Shannon,

estructura de la población (AMOVA) y se realizó un análisis de coordenadas principales (PCA). Se realizó un dendrograma UPGMA basado en clústeres de unión de vecinos.
Resultados: Se utilizaron cinco cebadores para generar 213 bandas polimórficas. La varianza molecular indicó un 73 % dentro de las localidades con un PhiPT (φ st) de 0.266 y una heterocigosidad esperada de 0.298 a 0.363. El PCA mostró cinco grupos diferentes: Noreste, Noroeste, Este, Centro y Suroeste. El AMOVA confirmó la asignación de los cinco grupos, pero el dendrograma ordenó los yacimientos en tres grupos: individuos del noreste y noroeste, individuos del centro y suroeste, e individuos del este.
Conclusiones: Los resultados sugieren que *H. alchorneoides* tiene una amplia distribución en Costa Rica y un flujo génico a larga distancia que mantiene la diversidad genética entre regiones. Se deben realizar más investigaciones para enriquecer la información producida en este estudio.

Palabras clave: AFLP, Phyllanthaceae, diversidad genética, pilón, bosque tropical lluvioso.

INTRODUCTION

Costa Rica is widely recognized for its great biodiversity, where wet forests constitute an important part of the country. Although Costa Rica has adopted conservation policies in past decades, neotropical forests have suffered historically deforestation and fragmentation due to human activity (Jeong et al., 2010; Jha & Bawa, 2006; Pennington et al., 2009; Raven et al., 2011). As a consequence, fragmentation of neotropical forests is frequently observed, which is accompanied by habitat fall or destruction, reduction of genetic diversity and in some extreme cases, the loss of a particular species, as a result of the reduced ability to reproduce or breed, and the lack of reaction mechanisms for natural selection or environmental adaptation (Bawa, Perry et al., 1985; Bawa, Bullock et al., 1985; Becerra & Venable, 2008; Fox & Reed, 2011; Hamrick & Godt, 1996; White et al., 2002). The genetic diversity and structure of plant populations depend on size, genetic migration, seed dispersal and pollination, mating systems and life cycle of a species (Dunphy & Hamrick, 2007; Hamrick & Godt, 1996).

In order to preserve and maintain endangered species of economic value, it is important to assess their population structure and genetic diversity. This will assist in identifying suitable populations as source material, which can be used in reforestation, reintroduction or management programs on wet forest (Clark & Clark, 2011; Clark et al., 2003; Clark et al., 2010; Hall et al., 2011; Park et al., 2010; Wishnie et al., 2007).

Hieronyma alchorneoides Allemao, is known as Pilón (Costa Rica), Bully Tree (Panamá), Nancitón (Nicaragua) and other names depending upon the country where it is found (Fournier & García, 1998; Jiménez-Madrigal et al., 1999). It is distributed from Belize to the Amazon region and the West of Indies. This species is a common tree in wet or rain forests and belongs to the family Phyllanthaceae (formerly classified within the family Euphorbiaceae, subfamily Phyllanthoideae), which comprises approximately 2 000 species in 59 genera, including herbs, shrubs, and trees (Hoffmann et al., 2006).

Several trees are of economic value, such as *Manihot esculenta*, *Hevea braziliensis*, *Ricinus communis* and *Croton* ssp. (Flores-Vindas, 1993).

H. alchorneoides is of wide distribution in Neotropic. Like most tropical trees, individuals of *H. alchorneoides* are distributed in large areas, in primary forests, national reserves, national parks or private areas. Within Costa Rica this species grows in Central, South Pacific, North and Atlantic Zone. It is a tall dioecious canopy tree, up to 50 m tall and diameter of 1-1.2 m, abundant in humid and very humid tropical forest, with an annual rainfall of 3 500-5 000 mm and altitude range from 20-900 m.a.s.l. This tree has two flowering periods: March to August and October to December and a fruiting period from November to July. *H. alchorneoides* is distinguished by the reddish-brown

stem and the reddish leaves. This species produces a strong, durable and very heavy wood, resistant to the attack of termites but it is susceptible to saprophytic fungi attacks, especially under ground level (Flores-Vindas 1993; Flores-Vindas, 2003). Wood is used in construction like houses, bridges, fenceposts and some fine carpentry. The tannin extracted from the timber is used for dyeing leather and there are also some reports from medicinal use of the seeds (Montero et al., 2007).

Despite being an economically important and threatened species, there are no other reported studies on *H. alchorneoides* genetic diversity using molecular approaches except for the one from Villalobos-Barrantes (2000), where three economical timber species, including *H. alchorneoides*, were analyzed with RAPD (Random Amplified Polymorphic DNA) approaches. In the study of Araya et al. (2005), they improve a DNA extraction method and report some combination of amplified fragment length polymorphism (AFLP) primers that show good quality bands, but this research lacks a genetic diversity approach. Other studies focus on physiological or ecological aspects about leaves, roots, fruits, propagation and wood (Abdelnour et al., 2011; Arnáez & Moreira, 2006; Montero et al., 2007; Moreira & Arnáez, 2009; Reich et al., 2004; Salazar & González, 2000) and there is one report on alkaloids content, extracted from *Hieronyma oblonga* (Alves & Zani**,** 1999). Clark and Clark have been studying several rain forest species including *H. alchorneioides* within La Selva Biological Station (Sarapiquí, Costa Rica) and its surroundings in a period of 10 years, they reported level of growth, adaptation to different types of soils and other data at ecological level (Clark et al., 2003; Clark et al., 2010; Clark & Clark, 1992; Clark & Clark, 1996). Given its economic importance for the wood quality that it presents, this species has been used in reforestation programs, especially in the Northern part of the country (Moya et al., 2009; Vallejos-Salazar et al., 2025).

DNA-based markers provide accurate and useful information to study genetic diversity in many plant species: for sample: *Coffea arabica* (Huang & He, 2010; Kafkas, 2006; Palacios et al., 2012; Ren & Timko, 2001; Steiger et al., 2002; Villalobos-Barrantes, et al., 2015), *Prunus avium* L. (Schueler et al., 2006), *Sideroxylon majus* (Dafreville et al., 2011), *Vochysia ferruginea* (Lowe et al., 2002) and *Pinus* (Liu et al., 2012; Navascués et al., 2006). The AFLP technique is very useful, robustness and powerful when compared to other techniques as RAPD or restriction amplified length polymorphism (RFLP) and microsatellites (Koopman, 2005; Koopman et al., 2007; Koopman et al., 2008; Vuylsteke et al., 2007): AFLP is i) applicable to all organisms without previous sequence information, ii) it provides the capability to detect various polymorphisms in different genomic regions simultaneously allowing the separation of closely related species and iii) it provides highly reproducible and robust data than other dominant multilocus marker systems as it generates a larger number of amplified products in a single reaction (Campbell et al., 2003; Savelkoul et al., 1999; Vuylsteke et al., 2007). This technique has been used to successfully study the genetic diversity in some tropical species (Andrade et al., 2007; Cavagnaro et al., 2006; Cavers et al., 2005; Lowe et al., 2003; Navarro et al., 2005; Reck et al., 2011; Soldati et al., 2013), including tropical trees (Hardesty et al., 2005; Kremer et al., 2005; Lowe et al., 2002; Lowe et al., 2003; Lowe et al., 2004; Lowe et al., 2018; Ward et al., 2005).

Recently, Pereira-Lima et al. (2022) classified *H. alchorneoides* as vulnerable timbre tree according to future climate change predictions in Brazil. Therefore, this study used the AFLP technique in *H. alchorneoides* to evaluate the genetic diversity of this species and provide information that can be used for conservation purposes.

MATERIALS AND METHODS

Plant material and DNA extraction: A total of 245 samples were selected from five regions within Costa Rica from primary and secondary forest: Northwestern (NO),

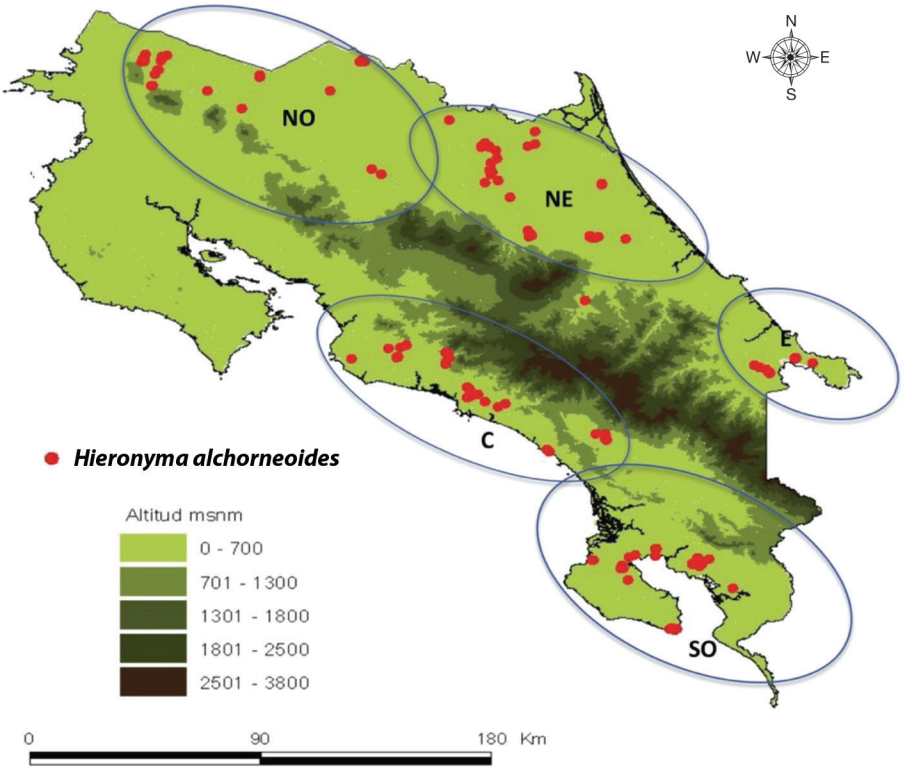


Fig. 1. Map showing sample locations of *H. alchorneoides.*

Northestern (NE), Central (C), East (E), Southwestern (SO) (Fig. 1). Samples were collected during January-March and July between 2005 and 2007 from adult trees with DAP > 0.70 m. Young leaves and cambium (when leaves were not accesible) were collected and kept on ice from the field to the laboratory and stored at -70 ºC until DNA isolation.

Total DNA was extracted from one gram of frozen leaves following the hexadecyltrimethylammonium bromide (CTAB) method (Doyle, 1990) with minor modifications. Briefly, leaves were ground with 7 ml of pre-heated CTAB buffer (100 mM Tris-HCl pH 7.6, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2 % CTAB, 1 % of β-mercaptoethanol, 0.01 % polyvinylpyrrolidone (PVP-40), 0.03 % of diethyldithiocabamic acid disodium salt trihydrate (DETC). Then samples were incubated at 55 ºC for 40 min, followed by extraction with 5 ml of chloroform-isoamyl alcohol (24/1) and centrifuged at 8 000 rpm for 25 min. Aqueous phase was recovered and mixed with 2/3 volume of cold isopropanol and incubated on ice for 20 min. The precipitated nucleic acids were recovered by centrifugation at 3 000 rpm for 30 min and dissolved in 400 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). A 200 µL of this solution was incubated at 37 ºC with 10 µg/ml of RNaseA for 30 min. followed by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Aqueous phase was recovered by centrifugation at 6 000 rpm for 5 min. A second extraction with an equal volume of chloroform-isoamyl alcohol (24/1) followed by a centrifugation at 6 000 rpm for 5 min. was performed. The recovered aqueous phase was mixed with 0.1 volumes sodium acetate (3M, pH = 5.2) and 0.67 volumes of cold ethanol (100 %) to precipitate the total DNA. The DNA

was recovered by centrifugation at 5 000 rpm for 8 min, washed with 70 % ethanol, dried and re-suspended in double distilled water. DNA quality was visualized on 0.8 % agarose gel electrophoresis stained with ethidium bromide. DNA concentration was calculated using a Beckman 750 spectrophotometer and adjusted to 200 ng for AFLP procedure.

AFLP protocol: AFLP technique described by Vos et al. (1995) was performed with minor modifications according to Prentis et al. (2004). Total genomic DNA, 200-500 ng, was digested with the restriction enzymes *Eco*RI and *Mse*I. The resulting fragments were ligated with the *Eco*RI and *Mse*I adapters. Pre-selective amplifications were performed using primers M-A, M-C, M-G and E-A, E-C in the following combinations: E-A/M-G, E-A/M-C, E-C/M-G, E-C/M-A. Final selective PCR reactions used *Eco*RI primers with two and three nucleotides and *Mse*I primers with three and four nucleotides in the following combinations: Eco+AA/Mse+GACC, Eco+AGC/Mse+GACC, Eco+CC/Mse+ACAG, Eco+CC/Mse+GACC, Eco+AGC/Mse+CAG. *Eco*RI primers were end labeled with Hex (Sigma Genosys, USA) and the rest of the oligos and unlabeled primers were obtained from Sigma Genosys (NSW, Australia). The amplification products were visualized by a 5 % polyacrylamide-7M Urea gel electrophoresis in a Gel Scan 2000 (Corbett Research, Australia).

Data analysis: Each AFLP product (60-400 bp) was assumed to represent a single biallelic locus, and data was manually scored as the present (1) or absence (0) of each band.

Allelic frequencies of AFLP loci were estimated using the Bayesian method with a non-uniform prior distribution of allele frequencies following (Zhivotovsky, 1999) and assuming no deviation from Hardy-Weinberg genotype proportions: *F* statistics and 99 % confidence intervals obtained by random permutation (9999 permutations) of individuals among regions, the proportion of polymorphic loci (PLP), the expected heterozygosity (He) or Nei´s gene diversity were calculated with the program ALFPSuv v. 1.0 (Vekemans, 2002; Vekemans et al., 2002). To test possible correlations between pairwise Fst and linearized geographic distance among populations, a Mantel test by random permutations (9 999 permutations) was carried out using GenAlEx v.6 (Peakall & Smouse, 2006). The Analysis of Molecular Variance (AMOVA) and the Phi values were also performed with GenAlEx. We used the Discriminant Analysis of Principal Components (DAPC) to evaluate the genetic structure in the library Adegenet (Jombart et al., 2010). We used cross-validation to determine the appropriate number of components to retain.

The Neighbor Joining Tree (NJ) tree of five populations was calculated using the program SplitsTree4 v. 4.11.3 (Huson & Bryant, 2006) based on genetic distance calculated by GenAlEx.

STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to estimate the number of genetically distinct populations (K). This approach is based on plotting the second-order rate of change in ln Pr (X/K) for successive Ks (referred to as ΔK) against a range of K values, and selecting the true K based on where the maximal value of this distribution occurs. We used the batch run function to carry out a total of 50 000 runs for a set of K from 1-9. Each run consisted of 5 x 10^5 interactions (Evanno et al., 2005; Gilbert et al., 2012; Pometti et al., 2012).

RESULTS

Five selected primers of AFLP combination were used in the analysis of *H. alchorneoides* samples that generated 213 bands from 245 individuals, of which the mean of polymorphic bands was 184 (87 % polymorphism). These primer combinations were chosen after a performed screening of 20 combinations, from which the most polymorphic ones were selected. Polymorphic bands have an average number of 211 fragments per individual but vary depending on the location (Table 1) and for each combination: Eco+AA/Mse+GACC generated 33 bands, Eco+AGC/

Mse+GACC 30 bands, Eco+CC/Mse+ACAG 53 bands, Eco+CC/Mse+GACC 47 bands, and Eco+AGC/Mse+CAG generated 50 bands. The expected heterozygosity (He) and Shannon's Information Index (I) were similar for each location (Table 1).

The dendrogram using NJ for the five locations showed two clades. One is gathering Northern populations (NO and NE respectively), another is gathering the C and SO and finally the E location in the middle of the clades. Northeast location is the most apart (Fig. 2).

Principal components analysis (Fig. 3) showed five discrete groups. The more compact groups were formed by the NE and C samples. The NO, E and SO groups were close to each other and some samples are mixed. The results showed a separation between the samples coming from the Northeast and central part of the country.

AMOVA analysis indicated that most of the genetic variation occurred within population (73 %) and the genetic differentiation (φst) was 0.266 ($p < 0.01$) (Table 2).

The Mantel Test indicated no clear correlation between genetic and geographical distances even when the geographical distance was linearized and the genetic differentiation (i.e.,



Fig. 2. UPGMA dendrogram showing the genetic relationships among the five locations of *H. alchorneoides* using AFLP datasets.

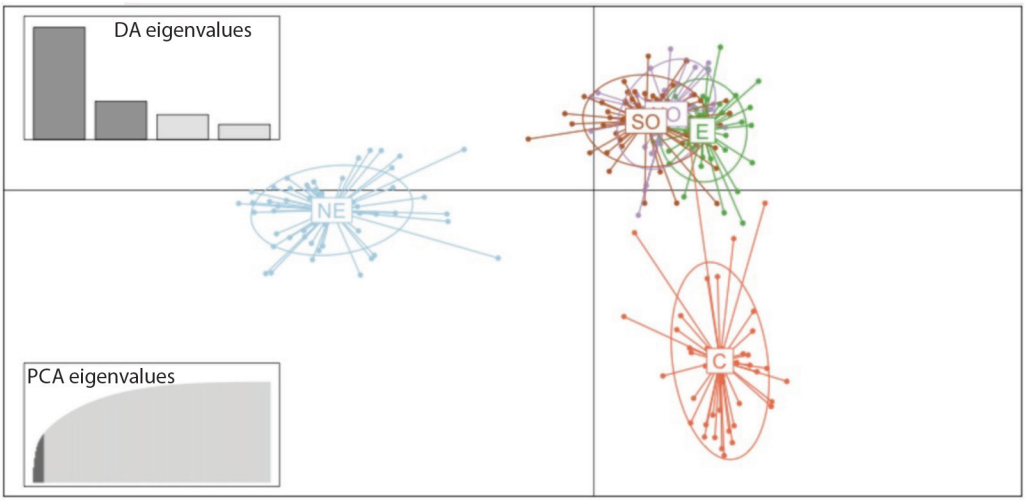


Fig. 3. DAPC analysis for 245 individuals of *H. alchorneoides* based on AFLP.

Table 1
Genetic diversity estimators for populations of *H. alchorneoides.*

Population	Size (n)	Total bands	Polymorphic bands	PLP	He	I
Northeast (NE)	62	213	198	93	0.349	0.405
East (E)	45	207	178	86	0.298	0.460
Central (C)	47	211	162	77	0.307	0.421
Northwest (NO)	34	211	194	92	0.363	0.481
Southwest (SO)	57	213	189	89	0.352	0.477
Mean						0.449
SE						0.006

PLP = percentage of polymorphic loci, He = Expected Heterozygosity, I = Shannon's Information Index.

Table 2
Analysis of molecular variance (AMOVA) based on AFLP analysis.

Souce	Df	SS	MS	Est. var.	%
Among populations	4	2 331.476	582.869	11.368	27
Within population	240	7 526.296	31.360	31.360	73
Total	244	9 857.771	614.229	42.728	100
PhiPT (φ st	0.266				
p	0.010				

p = p-value, PhiPT (φ st) = genetic differentiation.

Fst/(1-Fst) among population was used (R^2 = 0.2674, p = 0.068).

STRUCTURE analysis allowed us to identify several groups. The highest modal value of ΔK was K = 5, corresponding to the number of geographical regions. This result is in accordance with data obtained with the PCA analysis and the dendrogram, that are also grouped in five separate clusters. Even STRUCTURE results detected admixture individuals especially in the NO, SO and E populations and less in NE and C, as the PCA shows (Fig. 4).

DISCUSSION

With high rates of deforestation in recent years, the study of genetic diversity on tropical forests is still a valid and crucial matter in terms of lawmakers. Costa Rica has supported the establishment of forest plantations

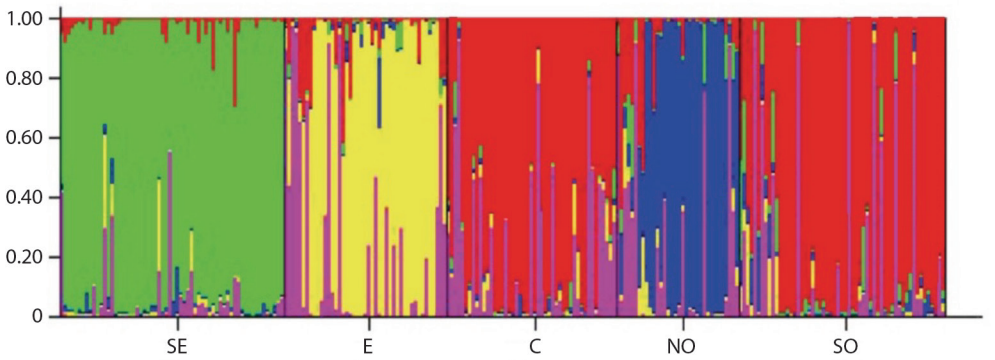


Fig. 4. Estimated population structure of *H. alchorneoides* inferred with STRUCTURE based on AFLP data. Populations are labelled below the figure according to the abbreviations used in Table 1.

or reforestation areas, especially for supplying with raw material the growing demand for wood and for environmental services such as carbon sequestration. These efforts have been complemented with reforestation programs with native flora in different parts of the country. In this way species as valuable as *H. alchorneoides* are being included in sustainable conservation programs since early 2000 (Calvo-Alvarado et al., 2007).

Most trees in tropical forests are vulnerable to the effects of habitat degradation because of low density, breeding systems, high rates of outcrossing pollinators and seed dispersal; this is particularly important for a dioecious and outcrossing species (Bawa, Perry et al., 1985; Bawa, Bullock et al., 1985; Dunphy & Hamrick, 2007; Fuchs et al., 2003; González-Zamora et al., 2012; Hamrick & Godt, 1996; Lowe et al., 2005; Trapnell & Hamrick, 2006).

This is the first comprehensive study throughout the natural distribution of *H. alchorneoides* in the country, which provides a complete view of its populations structure. We sampled individuals from five different regions within Costa Rica, comprised by primary and secondary rain forest and patches of forest. The heterozygosity and Shannon diversity index values in the five areas were similar, having the East site the lowest value (Table 1). These values are similar to other reported for tropical species, within a range between 0.1021-0.424 using AFLP (Cavers et al., 2005; Davies et al., 2010; Lowe et al., 2003).

AMOVA values showed a 73 % of the molecular variance within populations (Table 2) and a p-value of 0.266. Which suggests that inbreeding is not significant for this species. These values are common features in tropical outcrossing species, in other words, with large variance within populations and minor variance among populations. These values are related to seed dispersal, gene flow, matting system and pollinators; however, for *H. alchorneoides* until now there is not information on these important ecological features despite extensive reviews in these topics (Nathan et al., 2008; Ward et al., 2005). For this reason, we propose that *H. alchorneoides* is a tropical dioecious typical outcrossing tree.

Dendrogram supports the PCA and STRUCTURE result. In fig. 2 it is clear that the five sites are different, with NE population more apart but in the same clade with NO population. C and SO populations close to each other in another clade and E population apart. This dendrogram also agrees with the site's geographical allocations.

One possible explanation for these results are: i) the NE sample has only 20 individuals out of 62 that came from La Selva Biological Station, which is a primary rain forest and ii) it is possible that individuals sampled are genetically more similar and significantly contribute to this differentiation; and finally, iii) there is some weak isolation by distance within this group that is not reflected in the overall analysis.

Given these results, we assume that *H. alchorneoides* does not show a clear difference between the groups and hence it is not possible to make a Pacific-Atlantic differentiation like it was the case for *V. ferruginea* (Cavers et al., 2005), *Cordia alliodora* (Chase et al., 1995), and *Sideroxylon capiri* (Villalobos-Barrantes et al., 2015), as well as colonization from South America to Central America like in *Cedrela odorata* (Cavers et al., 2003). The results suggest that *H. alchorneoides* has a wide range distribution in Costa Rica with a long-distance gene flow that keeps maintaining the genetic diversity between regions, to counteract the effects of logging that enhance a forest fragmentation. The long-distance gene flow is a frequent phenomenon in tropical species (Dick et al., 2008; Hardesty et al., 2006; Hardesty et al., 2010).

Further research must be accomplished to enrich the information generated by this primary study. Like studies covering analysis of pollinators and seed dispersal. Also, complementary molecular markers like microsatellites can be used to corroborate values of genetic diversity generated in this investigation, to have a deeper population structure comprehension of this valuable native timber tree. Although with our results we can conclude that: i) AFLP

technique is a powerful and reliable procedure for assessing genetic variation among populations of a given species, as it was able to determine a differentiation between samples belonging to different regions and ii) our results provide elements to guidance to gene conservation actions and to support programs aiming to preserve specimen samples from different sites, given the similarity in genetic diversity among different regions. This feature is important when considering the expansion of conservation areas and the initiation of reforestation programs with native trees.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

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